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Genetic Counseling of Isolated Carriers of Duchenne Muscular Dystrophy

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It has recently become possible to detect female carriers of Duchenne muscular dystrophy with no affected male relative in the family. These "isolated carriers" represent about 10% of women with high serum creatine phosphokinase (CPK) levels and clinical evidence of a muscle disease. Most isolated carriers ascertained by clinical and/or CPK levels and diagnosed by dystrophin immunostaining of muscle biopsy show symptoms of a muscular dystrophy, and often carry the diagnosis of recessive "limb-girdle muscular dystrophy" prior to dystrophin analysis. It has been difficult to offer genetic counseling and prenatal diagnosis for Duchenne muscular dystrophy in the families of these isolated carriers, largely due to the difficulty in determining which of the dystrophin alleles segregating in the family harbors the mutation in the heterozygote. Here we report genetic counseling of three isolated carriers and their families. In two cases, prenatal diagnosis of at-risk pregnancies was conducted. We determined X inactivation patterns and inheritance of X chromosomes in each family, and used this information to define the at-risk dystrophin gene. In all three families, the mutation was a *de novo* event, two in the paternal germline, and one in the maternal germ-line. In each case we show that sibs of the heterozygous woman are at population risk, while pregnancies of each propositus are at high risk. Our results show that accurate genetic counseling and prenatal diagnosis can be offered to these families. © 1996 Wiley-Liss, Inc.

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INTRODUCTION

Duchenne muscular dystrophy is one of the most common genetic diseases that is lethal in childhood, and affects all world populations equally. It is an X-linked recessive disorder with a high sporadic mutation rate (1/10,000 gametes). The causative gene at Xp21 [Monaco et al., 1986; Koenig et al., 1987] is extremely large and its major product is "dystrophin" [Hoffman et al., 1987]. Dystrophin is a component of the membrane cytoskeleton, and is thought to impart structural integrity to the muscle [Hoffman and Gorospe, 1991].

In autosomal recessive conditions involving null mutations, heterozygous carriers will express half the protein product in each of their expressing cells. To the contrary, female carriers of X-linked disorders are most often "mosaics" due to X inactivation. Thus, if X inactivation is random and shows no preference for one or the other X chromosome, half the expressing cells should produce 100% of the protein product, while half produce 0%. This situation becomes more complicated with Duchenne muscular dystrophy due to the presence of additional variables. First, nuclei in muscle exist in syncytial myofibers, with thousands of nuclei sharing the same cytoplasm: dystrophin may diffuse from positive regions to negative regions in the same myofiber. Overproduction of dystrophin by nuclei with the normal X active is also possible: this "biochemical normalization" has been documented in mice [Watkins et al., 1989], dogs [Cooper et al., 1991], and humans [Pegoraro et al., 1995]. Second, regions of muscle fibers which are dystrophin-deficient are subject to segmental cell death. These same fibers can be regenerated by stem cells with the normal X active. This "genetic normalization" has recently been shown to have a major influence on the percentage of normal dystrophin genes active in carrier muscle [Pegoraro et al., 1995]. Genetic normalization leads to X-inactivation patterns in mus-

cle DNA which are substantially different from the patterns in lymphocytes in the same patient [Pegoraro et al., 1995]. Finally, X-inactivation patterns can often be "skewed," with the majority of cells inactivating the same X chromosome. We have found that isolated female carriers that show clinical symptoms typically have skewed X inactivation, in which the majority of cells have the X chromosome bearing the abnormal dystrophin gene active [Pegoraro et al., 1994].

It has been difficult or impossible to provide definitive genetic counseling to families of isolated female carriers of Duchenne dystrophy because there is no affected male relative to define the at-risk dystrophin gene. As such, it is often impossible to "set phase" between intragenic polymorphic markers and the dystrophin gene mutation. Bayesian risk estimates would indicate that parental risks for an isolated female carrier would be 50% chance that the mother is a carrier, 25% chance that the mutation is a new event from the mother's germ-line, and 25% chance that the mutation is from the father's germ-line [Pegoraro et al., 1994]. However, emprical studies have shown that about 90% of dystrophin gene mutations in isolated female carriers are inherited as a new mutation from the paternal germ-line [Pegoraro et al., 1994]. Deletion mutations are present in 54% of cases [Hoffman, 1993], and thus easy to diagnose by molecular techniques. The remaining cases have mutations not simply detected, and counseling must be based on probability adjusted for biochemical analysis. Isolated carriers of Duchenne dystrophy are not uncommon; about 10% of isolated cases of "limb-girdle muscular dystrophy" in females are really isolated carriers of Duchenne muscular dystrophy [Arikawa et al., 1991]. Further, about 10% of women with chronically elevated serum CPK in excess of 1,000 IU/l (nl <200 IU/L) are isolated heterozygotes [Hoffman et al., 1992]. Here we show that many of the previous difficulties in genetic counseling of these cases can be overcome by a combined molecular approach of quantitative assay of X-inactivation patterns, determination of inheritance patterns of skewed X inactivation, deletion analysis, and fluorescent multiplex linkage analysis of the dystrophin gene.

METHODS

Case Studies

Proposita, family 1. Dystrophin protein analysis and X-inactivation data has been previously described for this 31-year-old woman who sought evaluation for easy fatigue [patient 45 in Hoffman et al., 1992; patient 18 in Pegoraro et al., 1995] (II-2; Fig. 1). No weakness was found either by patient report or clinical examination, but multiple serum CPK measurements were found to be markedly elevated (4,284 IU/l, 1,071 IU/l, 2,227 IU/l; normal <200 IU/l). There was no family history of any neuromuscular disorder. A muscle biopsy showed minor histopathological changes consistent with a mild muscular dystrophy. Dystrophin analysis of the biopsy showed the mosaic pattern of positive and negative myofibers diagnostic of a carrier of Duchenne dystrophy. Quantitation of dystrophin by quadruplicate immunoblots showed a level of 62 ± 18% [Pegoraro et al., 1995]. There was no cytogenetic abnormality.

Proposita, family 2. This previously unreported 9-year-old girl was first seen at age 6 years for markedly elevated serum CPK levels (3,126 IU/l) detected incidentally during blood testing, but she was considered asymptomatic at that time (III-1; Fig. 2). A mild learning disability was noted. At age 8 years, neurological exam showed mild weakness in a limb-girdle distribution, and a muscle biopsy was done. The biopsy was not preserved well, however there was suggestion of numerous dystrophin negative fibers, and quantitative immunoblotting showed dystrophin to be approximately 70% of normal levels. There was no family history of neuromuscular disease, and the patient showed no cytogenetic abnormality by high resolution studies.

Proposita, family 3. This 10-year-old girl was reported by her parents to be well until age 9 years, whereupon she showed difficulty in getting up out of a chair, and stumbled easily when walking (II-1; Fig. 3). Neurological examination found hypertrophy of the calves, mild proximal weakness, and a modified Gower sign. Serum CPK was over 2,000 IU/l. There was no family history of neuromuscular disease. A muscle biopsy showed marked fiber size variation, central nuclei, some fibrofatty replacement, and degenerating and regenerating myofibers, consistent with a relatively severe muscular dystrophy. Dystrophin analysis showed clear mosaicism diagnostic of a carrier of Duchenne muscular dystrophy. Quantitative immunoblotting showed dystrophin at levels 16 6 9% of normal levels [patient 10; Pegoraro et al., 1995]. There was no cytogenetic abnormality by high resolution studies.

Molecular Studies

Dystrophin protein analysis of muscle biopsies was done as we have previously described [Hoffman et al., 1992]. Briefly, 4 μm cryosections were thawed on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA), and processed for dystrophin immunofluorescence using two polyclonal antibodies (30 kd [Hoffman et al., 1987], and d10 [Koenig and Kunkel, 1990]). Parallel cryosections were stained for routine histopathology by hematoxylin and eosin. The observation of populations of dystrophin-positive and dystrophin-negative myofibers by immunofluorescence were diagnostic of carriers of Duchenne muscular dystrophy.

Dystrophin gene haplotyping was done using a multiplex PCR reaction of four dinucleotide repeat loci distributed throughout the dystrophin gene [Beggs et al., 1990; Clemens et al., 1991; Feener et al., 1991], as we have previously described [Schwartz et al., 1992]. Samples were electrophoresed on a DuPont Genesis 2000 automated sequencer with molecular weight markers, lanes automatically tracked, and traces manually aligned to assign alleles.

X-inactivation quantitation and inheritance was done using PCR primers flanking the CTG repeat in the

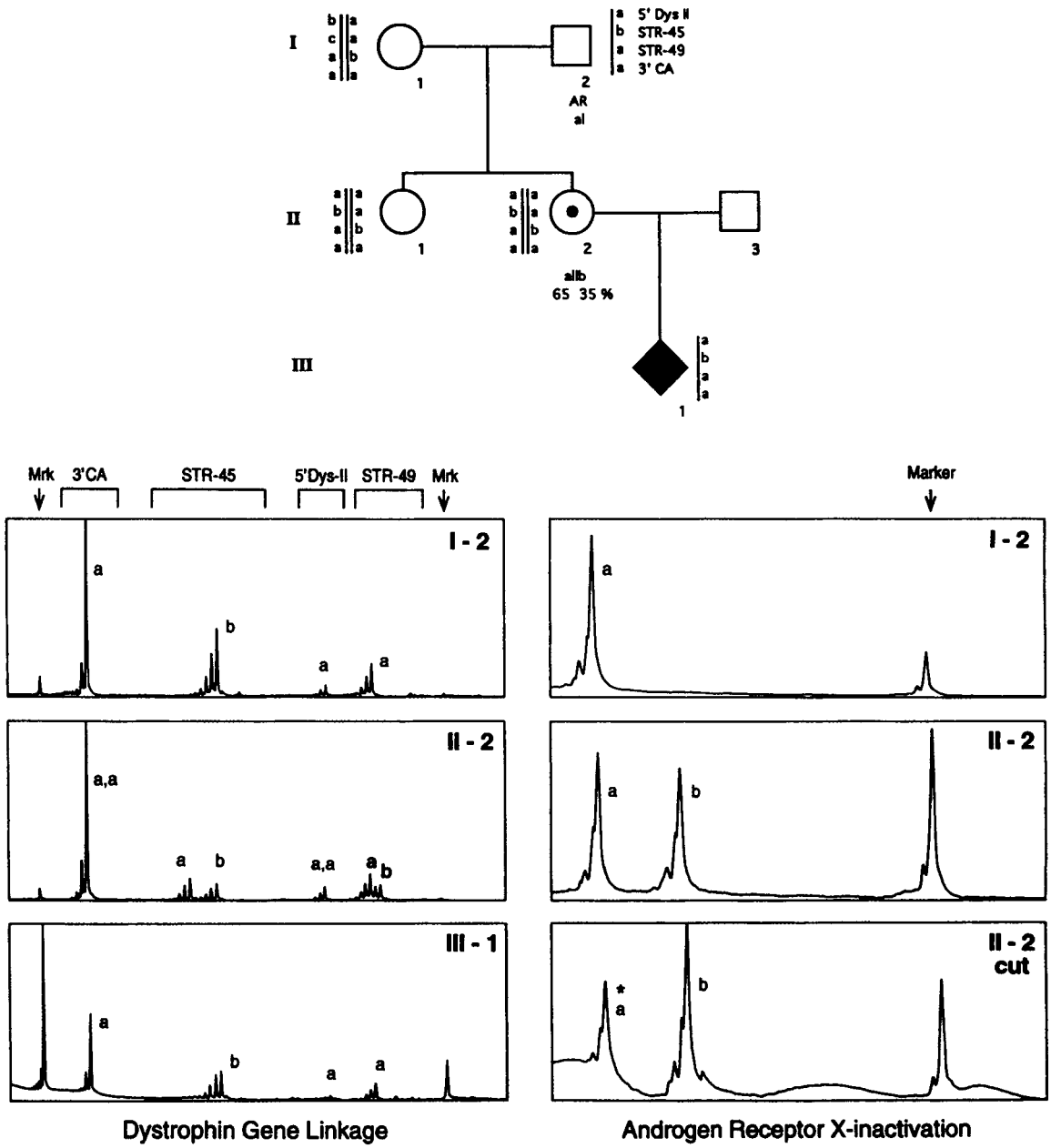
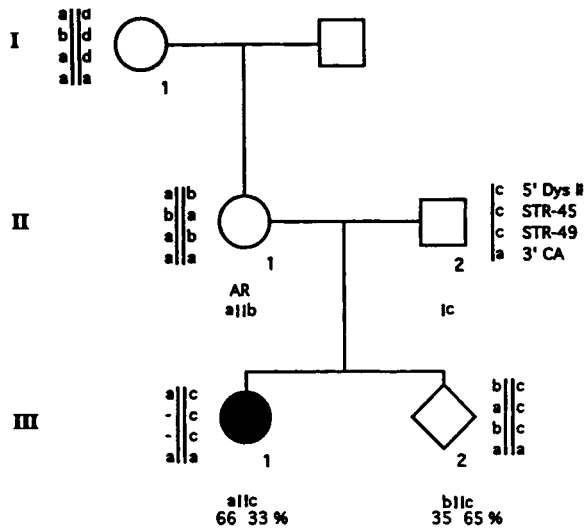
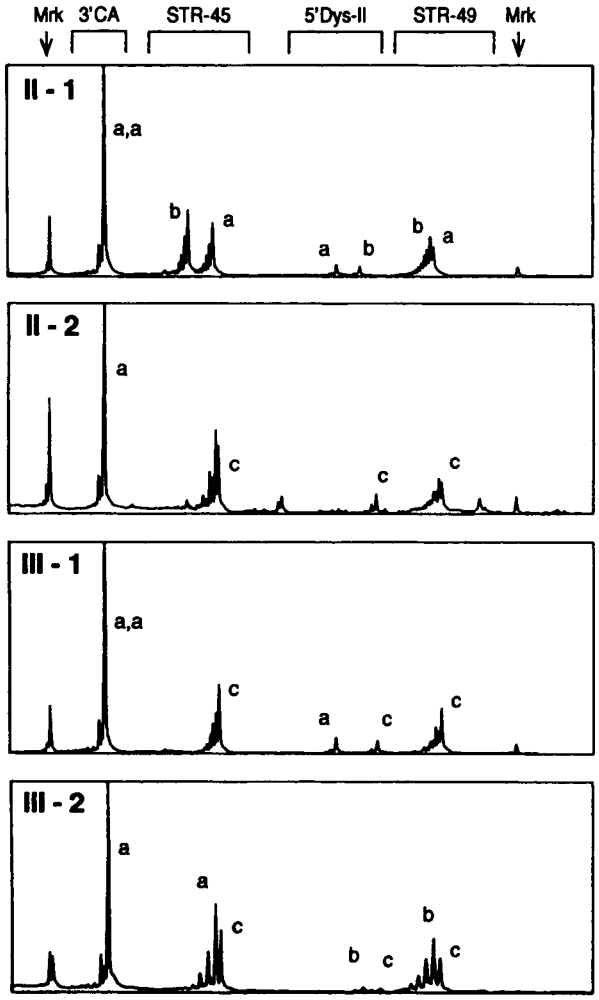
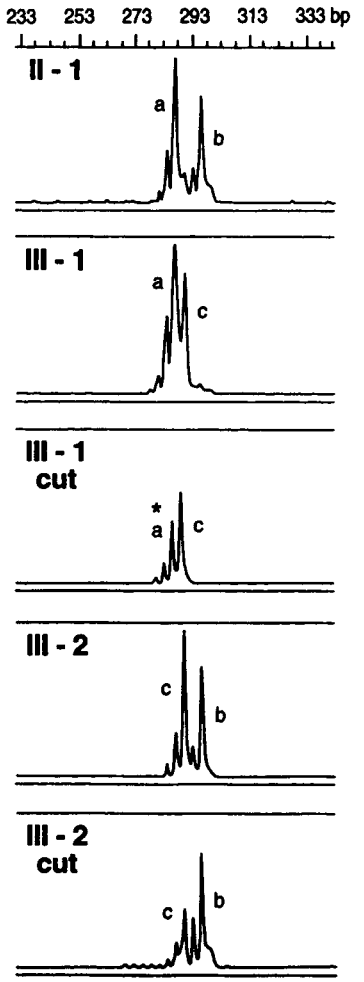


Fig. 1. Prenatal diagnosis in an isolated carrier detects an affected fetus with the grandpaternal dystrophin gene (family 1). Shown is the pedigree of the proposita/consultand (II-2), with data on X-inactivation patterns and inheritance using the androgen receptor (**right panels**), and dystrophin gene linkage using multiplex fluorescent dinucleotide repeats (**left panels**). The code for haplotypes of the dystrophin gene and androgen receptor are shown near individual I-2. The quantitation of percentage of each X active is shown below the androgen receptor alleles in individual II-2. Individual II-2 showed the paternal androgen receptor gene to be active in 65% of nuclei, suggesting that her grossly elevated serum CPK and easy fatigue were likely a consequence of a paternally derived de novo dystrophin gene mutation (right panels, "a" allele marked with an asterisk). The paternally derived dystrophin gene was defined in the proposita, and it was determined that the male fetus of the proposita inherited this at-risk dystrophin gene. The fetus was assigned a high relative risk (85%) for Duchenne muscular dystrophy but was carried to term. The term baby was a male with persistent serum CPK in excess of 19,000 IU/l and was diagnosed with Duchenne muscular dystrophy. Experimental data is shown for a subset of individuals, with all results summarized on the pedigree.





Dystrophin Gene Linkage



Androgen Receptor X-inactivation

Fig. 2.

promoter of the androgen receptor gene [Allen et al., 1992]. This assay was modified for quantitation on automated sequencers, as we have previously described [Pegoraro et al., 1994]. Amplification was done before or after digestion with methylation sensitive restriction enzymes (*Cfo*I, *Hpa*II), and products electrophoresed on either DuPont Genesis 2000 or ABI 376A automated sequencers. On ABI sequencers, internal ROX molecular weight standards were used (see Fig. 2). On DuPont machines, a single molecular weight marker labeled with fluorescein was used (see Figs. 1 and 3).

RESULTS

Family 1

The proposita (II-2) was a known isolated carrier of Duchenne muscular dystrophy based upon testing of muscle biopsy, and she presented for prenatal diagnosis of her first pregnancy (Fig. 1). Southern blot analysis of the dystrophin gene showed no alteration in dosage for any exon, suggesting that she did not have a deletion mutation. X-inactivation patterns in the proposita were determined through fluorescent PCR analysis of androgen receptor alleles, as previously described [Allen et al., 1992; Pegoraro et al., 1994]. This analysis showed the "a" allele of the androgen receptor to be preferentially active (65% of cells), and hence was likely in-phase with the mutant dystrophin gene given the proposita's very high serum CPK level. The "a" androgen receptor allele was inherited from her father (I-2) (Fig. 1, right panels). These data suggested that the proposita's abnormal dystrophin gene was paternally inherited as a de novo mutation. The accuracy of this interpretation is discussed later.

Multiplex fluorescent linkage analysis using CA repeats distributed throughout the dystrophin gene was then done on the family and the fetus (Fig. 1, left panels). This analysis defined the at-risk haplotype as "abaa," which was present in the proposita's father (I-2). The fetus was shown to be male and to have inherited the grand-paternal dystrophin gene haplotype. Thus, the fetus carried a very high risk for Duchenne muscular dystrophy (approximated as 85% based on unknown accuracy of the assays in cases with minor degrees of X-inactivation skewing). After considerable counseling and discussion including the option of fetal muscle biopsy to modify risks further, the proposita elected to continue the pregnancy (fetal muscle biopsy carries a 5–10% morbidity risk, although the analysis is considered quite accurate if the diagnosis of Duchenne dystrophy in male relatives is certain [Evans et al., 1991, 1994]). A male baby was born with persistently elevated serum CPK (160,000 IU/l at birth, 24,005 IU/l at 2 weeks, 19,570 IU/l at 5 months, 19,440 IU/l at 11 months; normal <200 IU/l). At birth the boy presented with a mild deformation of one side of the face and a small cardiac interventricular communication without clinical symptoms. Both corrected spontaneously over the next year. At 1 year of age he can stand without support, but cannot yet walk. It was concluded that the baby had Duchenne muscular dystrophy based primarily on laboratory findings.

The recurrence risk for the parents of the proposita (I-1, I-2) was determined to be that of paternal gonadal mosaicism (15%) [Bakker et al., 1989; van Essen et al., 1992]. The risk of the proposita's sister, II-1, of being a carrier was the paternal gonadal mosaicism risk (15%): any male pregnancies with the "abaa" haplotype would carry a 15% risk of having Duchenne muscular dystrophy.

The proposita recently became pregnant a second time. Dystrophin gene linkage studies showed a female fetus with the grandmaternal dystrophin gene. Thus, the fetus was diagnosed as a normal female. This pregnancy is ongoing.

Family 2

The parents of the proposita (III-1) sought genetic counseling for possible recurrence of "limb-girdle muscular dystrophy" in a current pregnancy. The muscle biopsy of the proposita was referred for dystrophin testing, which showed the girl to be a carrier of Duchenne muscular dystrophy: clear populations of dystrophin-negative and dystrophin-positive myofibers were seen by immunofluorescence [Hoffman et al., 1992]. X-inactivation patterns in the proposita (III-1) showed the maternally derived X to be preferentially active (66% maternal X active; 33% paternal X active), although the alleles were very closely spaced, making precise quantitation difficult (Fig. 2, right panels). This analysis suggested that the maternally derived dystrophin gene was the at-risk gene. Multiplex fluorescent CA repeat analysis of dystrophin gene polymorphisms showed that the proposita (III-1) had inherited a maternally derived de novo deletion mutation encompassing markers STR-45 and STR-49. Her mother (III-1) was not a carrier for this same mutation, because she was heterozygous at both of these loci (Fig. 2).

The fetus (III-2) was determined to be female, and was heterozygous at the STR-45 and STR-49 loci, indicating that she had not inherited the deletion mutation. Moreover, the female fetus did not share a maternal dystrophin gene with her affected sister. The female fetus also showed slight skewing of X inactivation, favoring activation of the paternal allele (Fig. 2, right panels), although this was within the range considered random X inactivation. This analysis showed that the female fetus was not a carrier of Duchenne muscular dystrophy.

Fig. 2. Counseling of the family of an isolated symptomatic girl carrier reveals a maternally derived de novo mutation, and a normal female pregnancy (family 2). Shown is the pedigree of the proposita (III-1), with dystrophin gene haplotypes and androgen receptor results summarized below tested individuals. The proposita shows preferential activation of the maternal X chromosome (right panels; "a" allele with asterisk). The close spacing of the AR alleles made quantitation difficult; however the maternal allele was approximated to be active in 66% of cells. The maternally derived dystrophin gene was found to contain a de novo deletion mutation encompassing CA repeat loci STR-45 and STR-49. This was not inherited by the female fetus, who was deemed to be a normal female. Experimental data is shown for a subset of individuals, with all results summarized on the pedigree.

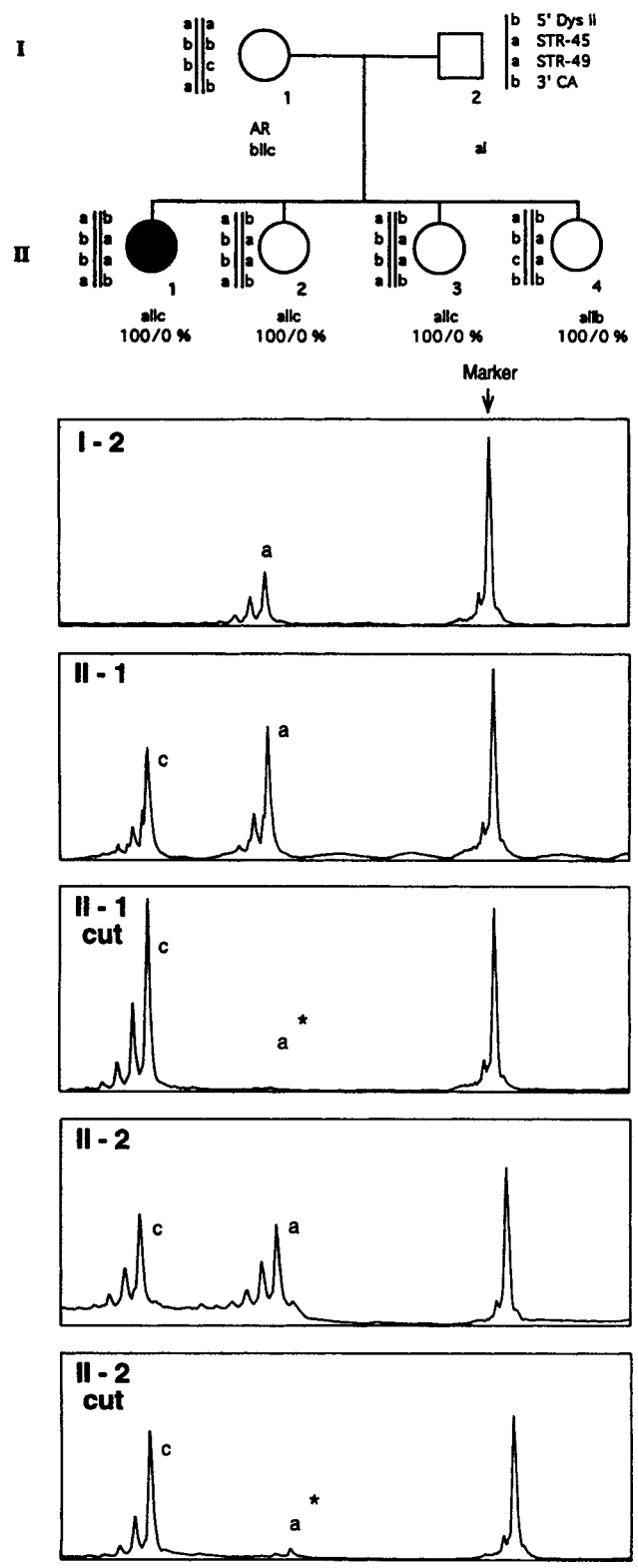


Fig. 3. Paternally derived de novo dystrophin gene mutation in a proposita (II-1) is probably not inherited by female sibs based on correlation of X-inactivation patterns and CPK data. Shown is the pedigree of a symptomatic isolated female carrier of Duchenne muscular dystrophy (II-1), with dystrophin gene haplotypes and androgen receptor X-inactivation data shown near each individual. The proposita is using the paternal X chromosome in 100% of cells, and shows marked dystrophin deficiency in muscle. This indicates a paternally derived new mutation. The a priori risk to her three female siblings for carrier status is the gonadal mosaicism risk to the father, or approximately 15%. However, each of the three sibs shows usage of the paternal X in nearly 100% of cells. If any of the three were in fact carriers, then they should show grossly elevated CKs and muscle symptoms. As all three had normal CPK, it was concluded that they were not carriers. Experimental data is shown for a subset of individuals, with all results summarized on the pedigree.

Family 3

The proposita (II-1) showed complete skewing of X inactivation, favoring the paternally derived X chromosome (Fig. 3). This analysis strongly suggested that the dystrophin gene mutation in the proposita was a paternally derived de novo event. Risk to future pregnancies of the proposita's parents are defined by the risk of paternal gonadal mosaicism (15%), and therefore only female progeny are at risk for being carriers.

The proposita had three sisters, and the a priori risk of being a carrier was 15% for each (risk of recurrence due to paternal gonadal mosaicism). To refine these risks, X-inactivation studies were done in each sister. All three sisters (II-2, II-3, and II-4) showed complete skewing of X inactivation, each favoring the paternal X (Fig. 3). Each daughter showed normal serum CPK, and no evidence of muscle weakness. This analysis suggests that each of the sisters is *not* a carrier of Duchenne muscular dystrophy.

DISCUSSION

The high frequency of Duchenne muscular dystrophy, and the large proportion of new mutations, serve to make isolated carriers a relatively frequent occurrence in the general population. Some isolated DMD carriers may be clinically detected due to expression of clinical symptoms (manifesting carriers), while others may be asymptomatic yet be detected with elevated serum CPK (hyperCKemia). While only 10% of females with hyperCKemia, with or without muscle weakness, can be shown to be isolated carriers of Duchenne dystrophy by muscle biopsy, the accuracy and specificity of the muscle biopsy test is thought to be quite high [Hoffman et al., 1992; Pegoraro et al., 1995].

Genetic counseling has been problematic in these cases: detection of deletion mutations in heterozygotes can be problematic, and interpretation of linkage studies is difficult if no male is available to define the at-risk dystrophin gene. Bayesian estimates have been at odds with empirical observations in the limited number of isolated female dystrophinopathy families studied to date: 90% of mutations in isolated carriers have been found to be paternally derived (27-fold variance with Bayesian predictions) [Pegoraro et al., 1994]. This finding further complicates genetic counseling and prenatal diagnosis.

Here we describe the use of X-inactivation skewing and inheritance patterns to define at-risk dystrophin genes, and then trace the at-risk gene to determine origin and assign risks to parents and sibs in the families. A critical assumption in this protocol is that the more

frequently active X chromosome contains the at-risk dystrophin gene. Our assays measured X-inactivation patterns in peripheral blood, and we then assumed that the X-inactivation patterns were similar in muscle tissue where dystrophin deficiency is defined. We have recently compared X-inactivation patterns in muscle and blood DNA in a series of female Duchenne dystrophy carriers and controls, and have found that blood DNA may in fact more accurately reflect skewed X-inactivation patterns present at the time of X inactivation in muscle, while muscle DNA changes with advancing age [Pegoraro et al., 1995]. This is because of the active genetic normalization processes in female dystrophinopathy patients which lead to a gradual decline in the extent of X-inactivation skewing with advancing age in muscle, but not in blood. In addition, we have recently completed an extensive comparison of X-inactivation patterns in blood DNA and oral epithelial cell DNA, and find that patterns are usually faithfully preserved between different tissue types that do not show selection due to dystrophin expression (Pegoraro, in preparation). Comparison of X-inactivation patterns in blood with clinical phenotype and biochemical data (extent of dystrophin deficiency in muscle) have also shown the expected correlations: the more severely skewed in blood DNA, the more severe the dystrophin deficiency, and the more severe the clinical phenotype [Hoffman et al., 1992; Pegoraro et al., 1995]. Given these observations, we believe that X-inactivation patterns in blood DNA permit assignment of the mutant dystrophin gene in these carrier females. It should be stressed that clinical, laboratory, and biochemical data must be studied and correlated carefully to check consistency with expectations.

In one of the three families presented here (family 3), the proposita showed 100% of blood cells using the paternal X chromosome (complete skewing). This, coupled with the severe dystrophin deficiency in her muscle (16% normal levels) and early onset of clinical symptoms, convincingly demonstrated that the dystrophin gene mutation was a de novo paternally derived event. Each of her three sisters has an a priori risk of being a carrier equal to the recurrence risk of gonadal mosaicism, which has been found empirically to be about 15% [Bakker et al., 1989; van Essen et al., 1992]. However, each of the proposita's sisters were also found to have complete or nearly complete skewing of X inactivation, each using the paternal X chromosome in ~100% of blood cells. If any of the three sisters were in fact carriers of the de novo paternal dystrophin gene mutation, then we would expect them to show clinical symptoms and grossly elevated serum CPK levels. As all three sisters showed normal serum CPK, we conclude that it is very unlikely that they are carriers.

In the other two families presented, the skewing of X inactivation was much less dramatic. In family 1, the proposita complained only of easy fatigue at age 25 years, yet had striking elevations of serum CPK (1,071–4,284 IU/l; nl <200 IU/l). As these CPK elevations are considerably higher than typically detected in asymptomatic female carriers with a positive family history at this age, we concluded that she likely had some degree of skewing of her X inactivation patterns. Quantitation of X inactivation in the proposita showed that 65% of blood cells were using the paternal X, and we tentatively assigned the paternal dystrophin gene as the at-risk gene. A subsequent pregnancy showed a male fetus with the at-risk grandpaternal dystrophin gene haplotype, suggesting that the fetus was affected with Duchenne muscular dystrophy. The fetus was carried to term and was indeed affected with Duchenne dystrophy. Although she clearly understood the severity of the disease, she opted to continue the pregnancy. In our experience with familial cases of Duchenne dystrophy, 5–10% risk is most often considered unacceptable by female siblings of affected males. The risks acceptable to patients vary depending on a wide variety of factors, including previous experience with the disease, religious and philosophical beliefs, and social and economic situation. While we present only a single example, this case suggests that individuals without direct experience of affected sibs with Duchenne dystrophy are likely to be more accepting of risk for having an affected child. This adds another level of complexity in the counseling of isolated female carriers of Duchenne dystrophy who do not have first-hand knowledge of the typical disease in males.

The analysis in family 2 was facilitated by the detection of a maternally derived de novo gene deletion mutation. The female fetus did not inherit the deletion mutation, and was deemed to be unaffected. Again, the X-inactivation patterns agreed with the maternal origin of the mutation, and with the relatively mild clinical and biochemical findings. The apparent ability to use X-inactivation patterns in blood DNA together with clinical and biochemical data to assign the at-risk chromosome can be contrasted to X-linked blood diseases where there is active selection against the cells with the mutant X active. In Duchenne dystrophy, where no such selection against blood cells exists, the *mutant* X is preferentially active in the majority of blood cells, while in disorders such as ADA the *normal* X is active in the majority of cells. It should be emphasized that the presence of skewing in peripheral blood cannot be used as evidence of a disease allele. Rather, once symptoms are present and biochemical (dystrophin) data obtained, skewing of X inactivation will suggest which chromosome contains the disease allele. This concept is reinforced by the four sisters in family 3.

Our results show that families with isolated female dystrophinopathy patients detected by muscle biopsy can be counseled successfully using a combination of clinical, biochemical, X inactivation, and dystrophin linkage data. It must be stressed that the number of cases studied to date is small, and thus it is as yet impossible to provide data on the accuracy of this testing protocol. It is also important to note that alternative procedures such as fetal muscle biopsy can be employed in pregnancies where the risk remains ambiguous after molecular testing [Evans et al., 1991, 1994; Kuller et al., 1992].

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